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The presence of 5-hydroxymethylcytosine at the gene promoter and not in the gene body negatively regulates gene expression

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ABSTRACT

5-Hydroxymethylcytosine (5hmC) was recently described as a stable modification in mammalian DNA. 5hmC is formed by the enzymatic oxidation of 5-methylcytosine (5meC). Overwhelming evidence supports the notion that 5meC has a negative effect on transcription; however, only recently has the effect that 5hmC has on transcription begun to be studied. Using model substrates including the CMV_{IE} promoter and a generic gene body we have directly assessed the effect that 5hmC, both at the promoter and in the gene body, has on *in vitro* gene transcription. We show that the presence of the 5hmC modifications strongly represses transcription. We also demonstrate that the inhibition of transcriptional activity is primarily due to the presence of 5hmC in the promoter and that 5hmC in the gene body has a minimal effect on transcription. Thus, we propose that the presence of 5hmC in promoter prevents the binding of essential transcription factors or recruits factors that repress transcription.

1. Introduction

Cytosine methylation in mammals occurs primarily at CpG dinucleotides. Cytosine methylation (5meC) is achieved by the enzymatic addition of a methyl group to the N5 position of cytosine. This enzymatic addition is specifically achieved by DNMT1 and DNMT3, which act to maintain DNA methylation or initiate *de novo* methylation. The presence of 5meC at gene promoters is a well-described mechanism of transcriptional regulation. In general, cytosine methylation at gene promoters causes a reduction of transcriptional activity (for a recent review see [1]).

In 2009 two independent laboratories discovered an oxidized form of 5meC – 5-hydroxymethylcytosine (5hmC) [2,3]. Initially, this modification was shown to be a stable DNA modification in embryonic stem cells and highly specialized Purkinje neurons [2]. This group was also unable to identify 5hmC in cancerous cell lines. Later, 5hmC was identified in all mammalian tissue studied [4–6]. Initial reports demonstrated that 5hmC resulted from the oxidation of 5meC catalyzed by the Tet1 protein [3]. Further research has shown that Tet1, Tet2, and Tet3 are capable of catalyzing the oxidation of 5meC resulting in 5hmC [7].

The 5hmC modification in mammalian DNA has been attributed to many cellular processes. Several reports have shown that 5hmC may be an intermediate in the oxidative demethylation of 5hmC [3]. 5hmC has also been implicated in early developmental programs [8] and necessary for stem cell renewal [7,9,10]. Deficiencies in Tet2 that led to aberrant 5hmC profiles have been implicated in the pathogenesis of certain myeloid tumors [11].

The genomic position of 5hmC suggests that 5hmC is found primarily in the body of the gene [11,12]; however, 5hmC may also be found in promoter regions [13]. Based upon current reports it has been suggested that 5hmC located in the gene body enhances transcriptional activity [8]; although, the presence of 5hmC in promoters appears to suppress transcription [9,14]. Some reports have suggested that 5hmC may relieve the transcriptional repression afforded by the 5meC modification [8,15].

The goal of this report is to directly address the effect that 5hmC has on transcription. Using an *in vitro* system we have been able to directly assess the singular effect that 5hmC has on transcription. We show, in a defined system, that the presence of 5hmC strongly suppresses transcription from the CMV_{IE} promoter. We also demonstrate that the inhibition of transcriptional activity is due to the presence of 5hmC in the promoter. Additionally, we show that 5hmC within a generic gene body has negligible effects on transcription.

Abbreviations: 5meC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; CMV_{IE}, cytomegalovirus immediate early promoter.

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2. Materials and methods

2.1. DNA substrates

Substrates were created by PCR amplifying the CMV_{IE} promoter and a generic gene body (sequence and PCR primers in Supplemental Fig. S1) with primers 1 and 2. Substrates containing 5hmC were amplified in the presence of d5hmCTP (Bioline) instead of dCTP. Substrates containing 5meC were incubated with 10 units M.SssI methyltransferase (NEB) in the presence of 160 μM S-adenosyl methylmethionine according to the manufacturer's instructions.

The substrate containing an unmodified promoter with a 5hmC gene body was created by the PCR amplification of the promoter with primers 1 and 3 and the PCR amplification of the gene body with primers 2 and 4 with d5hmCTP in place of dCTP. These PCR products were purified on a gel using a gel extraction kit (Qiagen) followed by digestion with *Sac*I (10 units/μg DNA) at 37 °C for 4 h. Enzymes were removed using a PCR clean kit (Qiagen). Four micrograms of 5hmC modified promoter DNA was ligated to 4 μg of cytosine gene body by incubation of the two DNA with 15 units T4 DNA ligase overnight at 16 °C. Correctly ligated (1.2 kbp) products were extracted from an agarose gel using a gel extraction kit (Qiagen).

The substrate containing a 5hmC promoter with an unmodified gene body was created by PCR amplification of the promoter with primers 1 and 5 with d5hmCTP in place of dCTP and the PCR amplification of the gene body with primers 2 and 6. These PCR products were purified on a gel using a gel extraction kit (Qiagen) followed by digestion with *Hind*III (10 units/μg DNA) at 37 °C for 4 h. Enzymes were removed using a PCR clean kit (Qiagen). Four micrograms of 5hmC modified promoter DNA was ligated to 4 μg of cytosine gene body by incubation of the two DNA with 15 units T4 DNA ligase overnight at 16 °C. Correctly ligated (1.2 kbp) products were extracted from an agarose gel using a gel extraction kit (Qiagen).

2.2. In vitro transcription reactions

Reactions (25 μl) containing 12 mM HEPES pH 7.9, 12% (v/v) glycerol, 0.3 mM dithiothreitol (DTT), 0.12 mM EDTA pH 8.0, 60 mM KCl, 3 mM $MgCl_2$, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 16 μM GTP, 10 μCi α-[^{32}P]-GTP, and 100 ng of DNA substrates as described above were initiated by the addition various concentrations of HeLa nuclear extract (Promega) and incubated at 30 °C for 60 min. The reactions were terminated by the addition of 175 μl of a solution containing 0.3 M Tris–HCl pH 7.4, 0.3 M sodium acetate, 0.5% (w/v) sodium dodecyl sulfate, 2 mM EDTA pH 8.0 and 3 μg/ml tRNA. RNA was extracted by phenol:chloroform extraction and ethanol precipitated. RNA pellets were dissolved in 98% formamide and electrophoresed on 6% (w/v) acrylamide gels containing 7 M urea. Electrophoresis continued until the bromophenol blue dye ran off the gel and exposed to a phosphorimaging screen. Results from each assay were normalized to the lowest concentration of nuclear extract using the substrate that contained unmodified cytosine.

3. Results

Recent reports have suggested that 5hmC may enhance or be inhibitory to transcription. The enhancement or inhibition of transcription by 5hmC has been attributed to the location of 5hmC either within the gene body or gene promoter, respectively [8–10,14,15]. We endeavored to elucidate the effect that 5hmC has on transcription using an CMV_{IE} promoter and a generic gene body

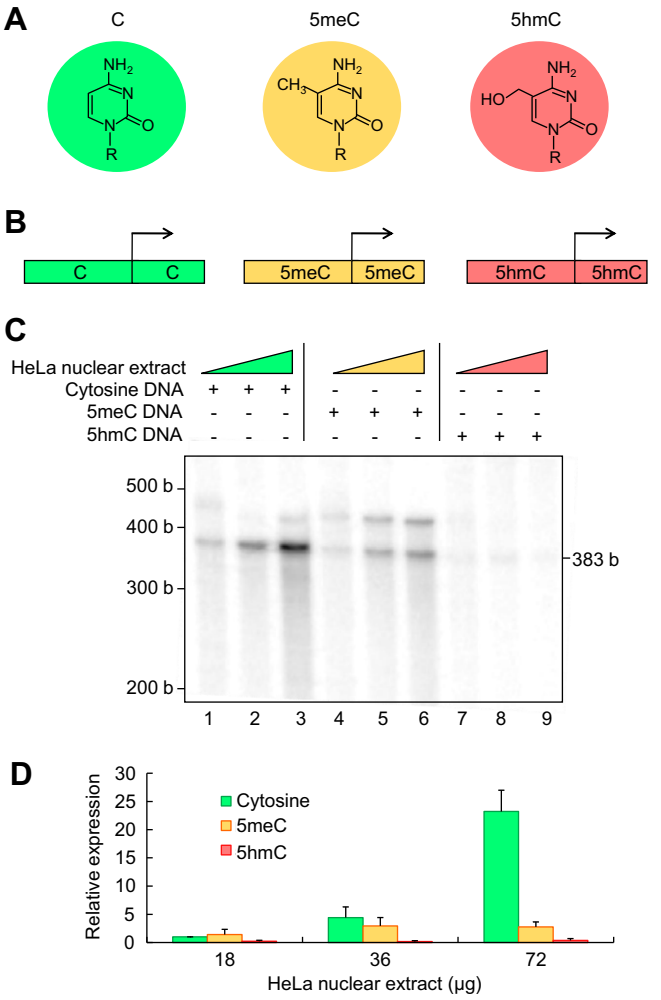

Fig. 1. 5hmC inhibits transcription from the CMV_{IE} promoter. (A) The modified bases used in this study. (B) The substrates used for the assay were synthesized as described in Section 2. (C) Increasing concentrations of HeLa nuclear extracts were incubated with 100 ng of a DNA substrate that was either unmodified, 5meC modified at CpG sequences, or all the cytosines were replaced with 5hmC, the band at 383 represents the run-off transcript from each DNA substrate. (D) Quantification of the transcripts synthesized by HeLa nuclear extracts from each substrate, error bars represent the standard deviation from the mean of at least three assays. Abbreviations: C: cytosine, 5meC: 5-methylcytosine, 5hmC: 5-hydroxymethylcytosine.

which allowed us to determine the precise effects that 5hmC has on transcription (Fig. 1A and B).

3.1. The presence of 5hmC in DNA inhibits transcription

We evaluated transcription activity using run-off transcription in HeLa nuclear extracts from the cytomegalovirus immediate early promoter (CMV_{IE}) followed by a 383 base pair sequence (Supplemental Fig. S1). Substrates were created using the DNA backbone shown in Fig. 1A that were either completely unmodified, 5meC modified at CpG regions, or every cytosine replaced with 5hmC (Fig. 1B). These substrates were incubated with increasing amounts of HeLa nuclear extracts. After incubation run off transcripts were isolated and identified using denaturing PAGE. Relative transcription was quantified as described in Section 2. After quantification we show that in a concentration dependent manner nuclear extracts from HeLa cells efficiently support transcription from the substrates that contain unmodified cytosines and, as expected, lower levels of transcription from DNA that contained 5meC modified at CpG regions. However, HeLa nuclear extracts were poorly able

to support transcription from substrates that contained 5hmC in place of cytosine (Fig. 1C and D). These results, taken together, strongly suggest that 5hmC is inhibitory to transcription.

3.2. 5hmC at the promoter and not in the gene body inhibits transcription

The above result strongly supports the hypothesis that the presence of 5hmC inhibits transcription; however, we wanted to address whether the presence of 5hmC in the promoter or in the gene body is inhibitory to transcription. To address this issue we designed hybrid substrates that contained cytosine in the promoter and 5hmC in place of cytosine in the gene body or 5hmC in place of cytosine in the promoter and cytosine in the gene body (Fig. 2A). These substrates were incubated with increasing amounts of HeLa nuclear extracts and the amount of run off transcript was quantified as described in Section 2. After quantification the substrate that contained 5hmC at the promoter with an unmodified gene body showed almost no run-off transcript whereas the substrate with an unmodified promoter and 5hmC in the gene body showed significantly higher levels of transcription (Fig. 2B and C). However, the levels of transcription from the unmodified promoter with a 5hmC gene body produced transcripts that were about three fold lower than substrates that contained only cytosine (Figs. 1D and 2C). These results support the notion

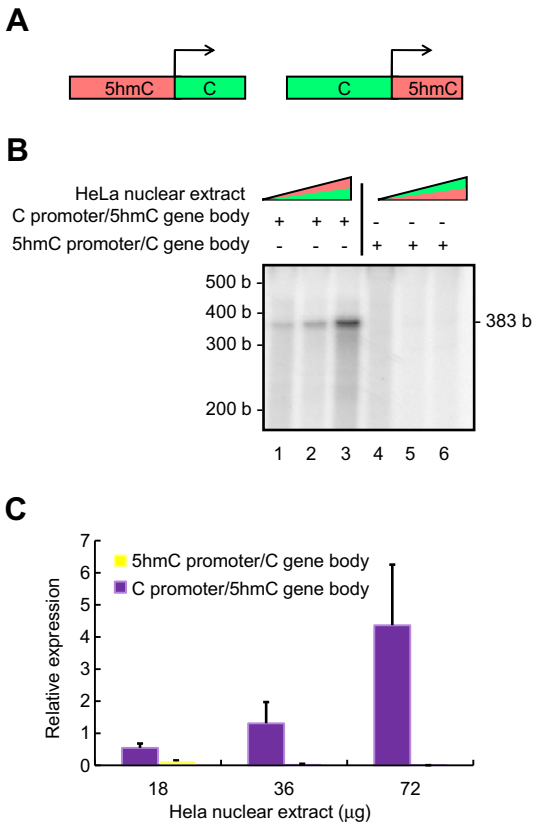

Fig. 2. 5hmC at the promoter is inhibitory to transcription. (A) The C promoter/5hmC gene body substrate represents an unmodified promoter with a 5hmC modified gene body; the 5hmC promoter/C gene body represents a substrate with a 5hmC modified promoter with an unmodified gene body. Substrates were synthesized as described in Section 2. (B) Increasing concentrations of HeLa nuclear extracts were incubated with 100 ng of the C promoter/5hmC gene body DNA substrate or the 5hmC promoter/C gene body DNA substrate, the band at 383 represents the run off transcript from each DNA substrate. (C) Quantification of the transcripts synthesized by HeLa nuclear extracts from each substrate. Error bars represent the standard deviation from the mean of at least three assays. Abbreviations: C: cytosine, 5meC: 5-methylcytosine, 5hmC: 5-hydroxymethylcytosine.

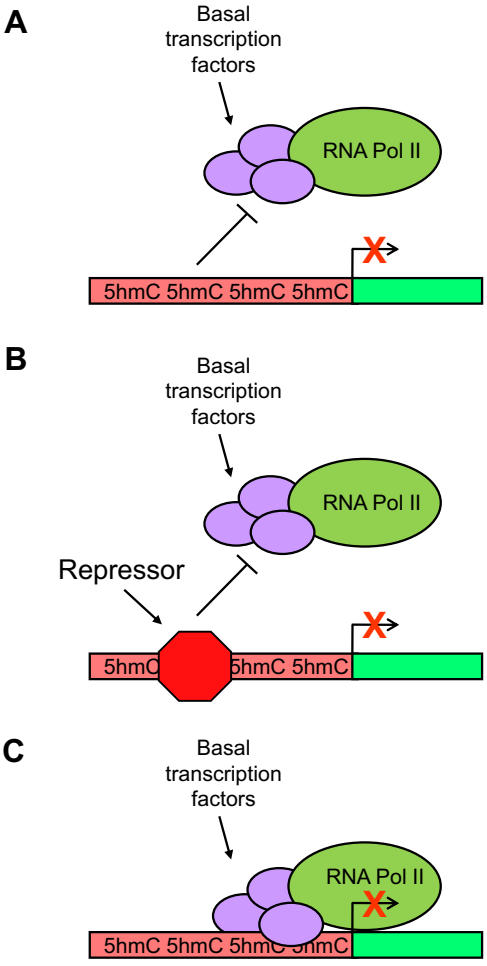

Fig. 3. Models representing the potential mechanisms of transcriptional inhibition from promoters that contain 5hmC modifications. (A) 5hmC at the promoter prevents the binding of basal transcription factors or RNA polymerase II effectively suppressing transcription. (B) 5hmC recruits another cellular factor(s) that prevents some or all of the transcription machinery from binding to the promoter suppressing transcription. (C) 5hmC at a gene promoter may allow for the basal transcription machinery to bind to the promoter; however, the 5hmC induces a conformational change in the basal transcription factors or recruits another protein that prevents the release of RNA polymerase II from the promoter inhibiting transcription initiation.

that 5hmC in promoters is inhibitory to gene transcription whereas 5hmC in the gene body does not directly inhibit transcription. However, 5hmC in the gene body does appear to reduce transcription, perhaps by reducing the transcription rate of RNA polymerase II. Additionally, this data suggests that 5hmC within the gene body does not by itself increase transcription.

4. Discussion

The effect that 5meC has on transcriptional regulation has been extensively studied; however, the effect that 5hmC has on transcription has only been indirectly addressed [8–10,14]. We aimed to directly evaluate the effect that the presence of 5hmC has on *in vitro* transcription using the CMV_{IE} promoter and a generic gene body in a well-defined mammalian system.

In this report we have directly demonstrated that the presence of 5hmC at the CMV_{IE} promoter strongly inhibits transcription in human nuclear extracts. This finding is not surprising given that two groups have indirectly shown that the presence of 5hmC at gene promoters reduces gene expression [10,14]. We also demon-

strate that the presence of 5hmC in the gene body has negligible effects on transcription in this *in vitro* system, contrasting with a previous report that suggests that 5hmC in the gene body increases transcription [8]. We have previously shown that the presence of 5hmC at the promoter may differentially affect gene transcription [13]. These previous studies used a less well-defined system that likely had 5hmC-modified DNA in association with histones that may, themselves, differentially affect transcription in the presence of 5hmC. The presence of histones in these studies makes it difficult to isolate singular function of 5hmC on gene transcription. The aim of our study was intended to measure the direct effect that the presence of 5hmC has on transcription in the promoter and/or in the gene body in the absence of nucleosomes. Demonstrating that 5hmC in the gene body has a limited effect on transcription is important because this finding rules out that 5hmC provides an elongation block to RNA polymerase II. Therefore, we can conclude that elongation catalyzed by RNA polymerase II is not inhibited by the presence of 5hmC and thus 5hmC prevents transcriptional initiation.

We propose that the inhibition of transcription initiation from a gene that has 5hmC at its promoter may occur by three different mechanisms. First, the 5hmC at the promoter may directly prevent the binding of necessary transcription factors or RNA polymerase II from binding to the DNA preventing the formation of the pre-initiation complex (Fig. 3A). Secondly, 5hmC may recruit an unknown factor to the promoter that inhibits the binding of basal transcription factors or RNA polymerase II (Fig. 3B). Currently, we are designing assays to assess the mechanism by which this transcriptional inhibition is carried out. Finally, 5hmC at the promoter may allow for the binding of the basal transcription factors and RNA polymerase II; however, 5hmC may provide an inhibitory signal to the pre-initiation complex preventing the initiation of transcription despite the presence of the all the necessary transcription factors and RNA polymerase II (Fig. 3C).

In summary, we have directly demonstrated that 5hmC located in the promoter region provides a significant inhibition to transcription. This inhibition is likely caused by 5hmC interfering with the assembly of the transcriptional pre-initiation complex or inhibiting the pre-initiation complex from releasing RNA polymerase II starting gene transcription.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.077.

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